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D-ring modified novel isosteviol derivatives: Design, synthesis and cytotoxic activity evaluation

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ABSTRACT

A series of polyhydric, amino alcohol and tricyclic derivatives were facilely synthesized by D-ring modification of isosteviol. These compounds were screened for their cytotoxic activities against four human tumor cell lines in vitro. Among them, the 15-α-aminomethyl-16-β-hydroxyl isosteviol **23** exhibits significant cytotoxicity superior to the positive control (cisplatin) against EC9706, PC-3 and HCT-116 cell lines.

The investigation aimed at discovering anti-carcinogenic compounds has attracted considerable interest in recent years since cancer is a leading cause of death worldwide. Natural products have always played a major role in anticancer medicine[1–3] and the unique metabolites produced by diterpenoids have increasingly become major players in antitumor drug discovery.[4–6] Isosteviol is a tetracyclic diterpenoid with a beyerane skeleton, obtained by acid hydrolysis of stevioside.[7] More and more researches in recent years showed that isosteviol as well as its derivatives possess a remarkably broad spectrum of biological activities.[8–15]

Previous reports proved that modifications on D-ring of the isosteviol skeleton could change its biological activities or lead to new activities.[16] Zhang and co-workers[17] reported that *exo*-methylene cyclopentanone sub-structure in the D-ring of tetracyclic diterpenoids could conduce noteworthy cytotoxicity superior to adriamycin. Nguyen's group[18] found two new symmetric dimers of *ent*-kaurane diterpenoid with the connectivity at ring-D from *Croton tonkinensis* showed a potent anti-tumor activity. Our previous study also showed that the C-15 and C-16 functionalized isosteviol had good cytotoxic activities against B16-F10 melanoma cells.[19] In addition, some work also indicated that biological activities of terpenoids would be improved by the introduction of hydroxyl groups through chemical synthesis[20] or microbial transformations.[21–23]

It is also well known that amino alcohols are important structural fragments of many bioactive compounds,[24–26] such as Naltrexone (Fig. 1, Compound (**A**)) and their derivatives.[27] Taneja et al. reported that the cytotoxicity was greatly improved when amino alcohol fragment was introduced to the ring-A of boswellic acids (Fig. 1, Compound (**B**)).[28] However, few reports have focused on the activity relationship of amino alcohol substituted isosteviol. Moreover, compounds with tricyclic skeleton are widely found in natural products, and some of them are proved to be good antitumor agents.[29] Isosteviol, an *ent*-beyerane type tetracyclic diterpene, can be converted to tricyclic compounds by D-ring opening reaction and showed excellent inhibition constants.[30] On the basis of these results, we designed and synthesized a series of novel polyhydric, amino alcohol and tricyclic derivatives via D-ring modifications of isosteviol for the purpose of discovering new antitumor active compounds.

Initial synthetic efforts were focused on novel polyhydric isosteviol derivatives and the synthetic routes are depicted in Scheme 1.

Esterification of compound **1** with CH_3CH_2Br and KOH in DMSO afforded the corresponding isosteviol ethyl ester **2** in high yield,

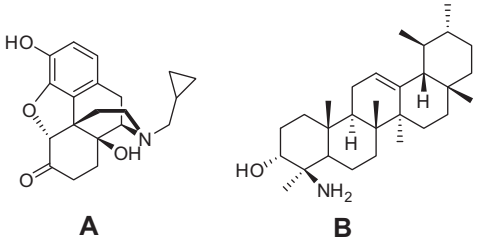

Figure 1. Structure of Compound **A** and **B**.

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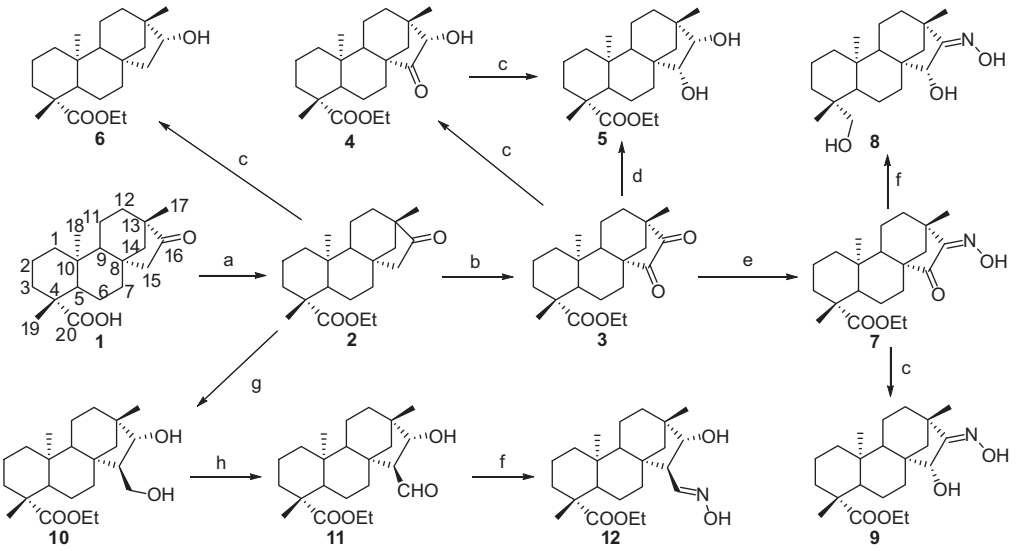


Scheme 1. Reagents and conditions: (a) EtBr, KOH, DMSO, rt, 3 h (95%); (b) Ac_2O, SeO_2, reflux, 6 h (83%); (c) 1.2 equiv. $NaBH_4$, MeOH, 0 °C; (d) 3.0 equiv $NaBH_4$, MeOH, 0 °C to rt, 2 h (90%); (e) $NH_2OH{\cdot}HCl$, $NaHCO_3$, EtOH, reflux; (f) 2.5 equiv. $LiAlH_4$, THF, reflux (80%); (g) HCHO, C_2H_5ONa, C_2H_5OH, 60 °C, 3 h (92%); (h) TEMPO, NBS, CH_2Cl_2/H_2O, TBAB, reflux (90%).

whereas only very low yields of **2** were obtained while using the classical esterification methods. Compound **2** was converted to **6** easily under $NaBH_4$/EtOH conditions.[31] The 15,16-diketone **3** was obtained as an orange crystal by the oxidation of **2** with selenium dioxide in acetic anhydride under reflux condition in high yield. Reduction of **3** with 1.2 equiv. or 3 equiv of $NaBH_4$ in methanol in ice bath gave uniquely the mono reduced product **4** or the 1,3-diol **5**, respectively. But a mixture of **4** and **5** were obtained when the amount of $NaBH_4$ was from 1.2 to 3 equiv. Compound **5** could also be prepared by the reduction of **4** with $NaBH_4$. The absolute configurations of the two newly formed chiral centers at C-15 and C-16 in compound **5** were identified by X-ray crystallographic analysis and the two hydroxyl groups located at *endo* position unambiguously (Fig. 2).[33] The steric effects of C10-CH_3, C13-CH_3 and ring carbon may be the reason to direct the approach of H^- onto the two carbonyls easily from the *exo* positions.

It is well known that oximido groups are usually considered as bioactive elements in biological studies. Therefore, one of the two carbonyls in compound **3** was designed to be converted to oximido following the introduction of a hydroxy unit by reduction of the other carbonyl group for the purpose of investigating the structure–activity relationship. After treatment of **3** with hydroxylamine hydrochloride and $NaHCO_3$ in ethanol under reflux

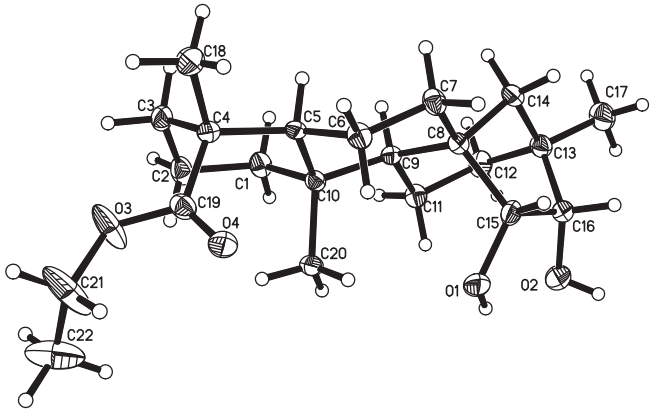


Figure 2. X-ray structure of compound **5**.

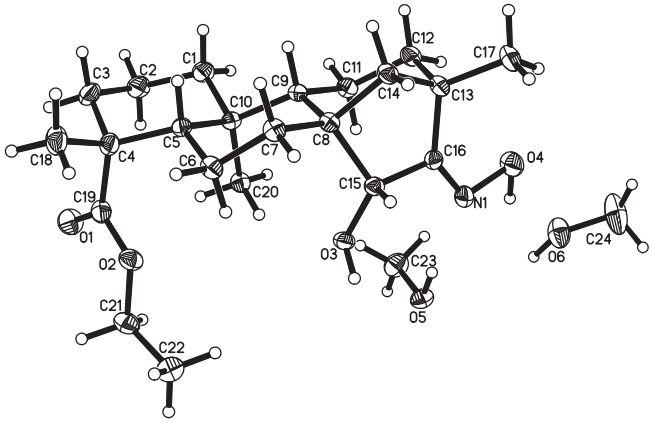
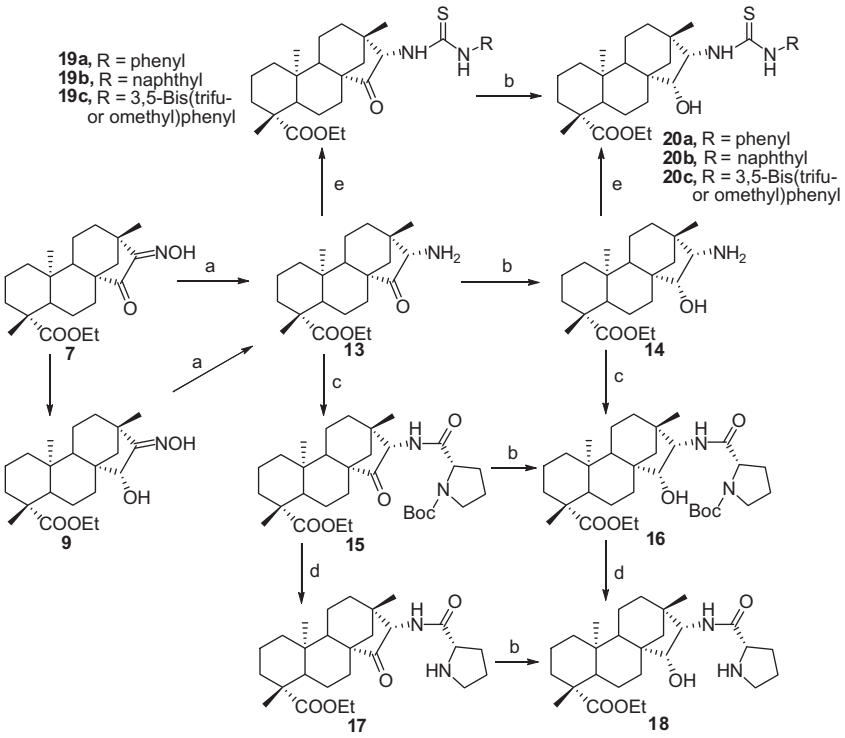


Figure 3. X-ray structure of compound **9**.

condition, only the 15-ketone-16-oxime derivative **7** was obtained as a yellow crystal. But attempts to obtain the 15-oximed derivative failed to meet our expectation. Then we decided to convert the carbonyl group at C-15 and the ester group at C-20 of compound **7** to hydroxyl groups in order to construct multi hydroxyl derivatives. When excess amount of $LiAlH_4$ in THF was employed as reducing agent, compound **8** was obtained as a white solid, whereas $NaBH_4$ could only reduce the C-15 carbonyl to give compound **9** as the unique product in high yield. The NOESY spectrum of compound **8** suggests that the C-15 hydroxyl group was at *endo* configuration. The absolute configuration of compound **9** was confirmed by the X-ray structure (Fig. 3),[33] indicating an *endo* direction of the hydroxyl group at C-15.

Moreover, in our previous work, compound **10** could be successfully achieved through a one-pot 'Aldol-Cannizzaro reaction' process,[32] which promoted us to probe 1,3-diol and its derivatives for the evaluation of their antitumor activities. Firstly, a clean and convenient TEMPO catalyzed oxidization of **10** gave **11** in 90% yield. Followed oximation reaction of **11** with hydroxylamine hydrochloride in the presence of $NaHCO_3$ in ethanol, compound **12** was furnished (87%).

The following work was focused on the introduction of amino and related functional groups onto the D-ring of isosteviol. A series



Scheme 2. Reagents and conditions: (a) Raney Ni/H_2 50 psi, 50 °C, THF, 5 h (95%); (b) $NaBH_4$, MeOH, 0 °C to reflux (80–85%); (c) *N*-Boc-L-proline, DCC, DMAP, DCM, rt, 6 h (90–92%); (d) CF_3COOH, DCM, 3 h, rt, (87–90%); (e) isothiocyanate, DCM, rt, 2 h (>97%).

of β-amino alcohol derivatives were firstly synthesized via stereoselective reduction of carbonyl and oximido groups in compound **7**. The synthetic approach employed to prepare 1,2-amino alcohol and their derivatives were outlined in Scheme 2.

Catalytic hydrogenation of the 15-ketone-16-oxime isosteviol derivative **7** with Raney Ni/H_2 in ethanol or isopropanol gave compound **13** in 95% yield. Reduction of **13** with $NaBH_4$ in anhydrous methanol generated amino alcohol **14** stereoselcetively as a white powder. Interestingly, when compound **7** was reduced to compound **9** by $NaBH_4$ firstly, then hydrogenation of **9** with the Ni catalyzed hydrogenation condition, the corresponding 1,2-amino alcohol **14** was not furnished, but unexpectedly, compound **13** was isolated in 96% yield. The conversion of **9**–**13** must undergo an imine rearrangement process.

Succeedingly, in order to further screen the structure-bioactivity relationship of the amino derivatives, we decided to convert the 16C-amine to its corresponding amide or thiourea. Reaction of **13** or **14** with *N*-Boc-L-proline and DCC in the presence of DMAP in CH_2Cl_2 furnished **15** and **16**, 'respectively'. Then treatment of **15** and **16** in TFA/DCM system generated the corresponding deprotected prolinamide **17** and **18**. thiourea derivatives were synthesized. In addition, reaction of **13 (14)** with isothiocyanate in DCM at room temperature provided corresponding thiourea derivatives **19** (**20**) in almost quantitive yield. The 15-carbonyl could be stereoselectively converted to hydroxyl group by reduction with $NaBH_4$ in methanol (**15**–**16**, **17**–**18**, and **19**–**20**).

Although the attempts to get the single crystals of **14** or its derivatives were failed, the *S*-configuration of the amino group at C-16 can be determined by the X-ray diffraction of **17** (Fig. 4).[33] The presence of NOE correlation of C-15-H and C-16-H indicating that the two hydrogen atoms were at the same direction and the hydroxyl and the amino groups were both at *endo* position (Fig. 5). The configuration of C-15 and C-16 were further confirmed by the observation of NOE effect between C-14-H and C-15-H (C-14-H and C-16-H, C-16-H and C-17-H). The structures suggested that the steric effect of C-10-CH_3, C-13-CH_3 and ring carbon make for an easy approach of nucleophile to C-15 and C-16 from *exo* position, while hard from *endo*.

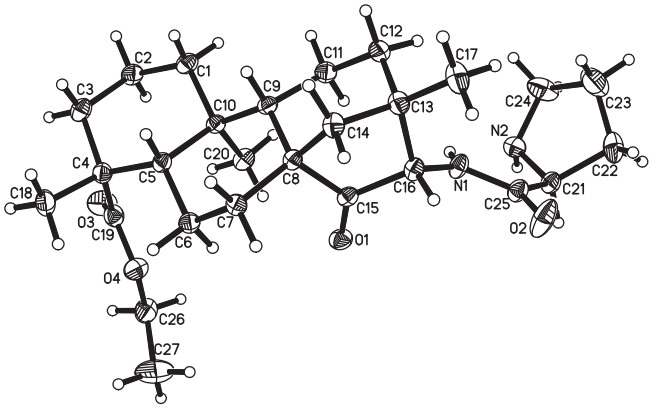


Figure 4. X-ray structure of compound **17**.

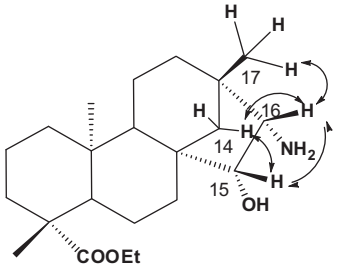
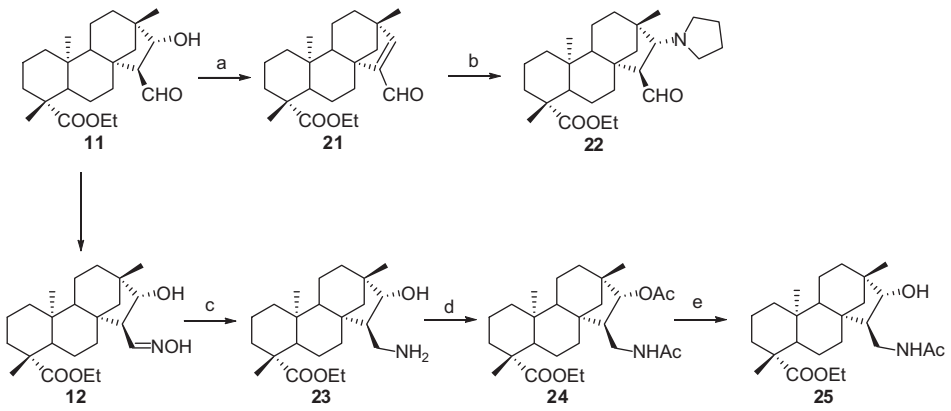


Figure 5. NOE correlations for compound **14**.



Scheme 3. Reagents and conditions: (a) DBU, pyridine (83%); (c) pyrrolidine, NaH, TBAB, DMF, rt (70%); (c) Raney Ni/H_2, 50 psi, 50 °C,THF, 5 h (80%); (d) Ac_2O, pyridine, rt, 3 h (90%); (g) methanol, K_2CO_3, rt (92%).

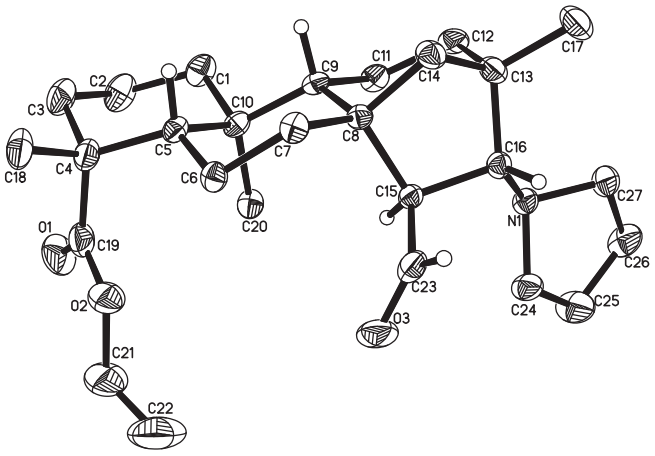


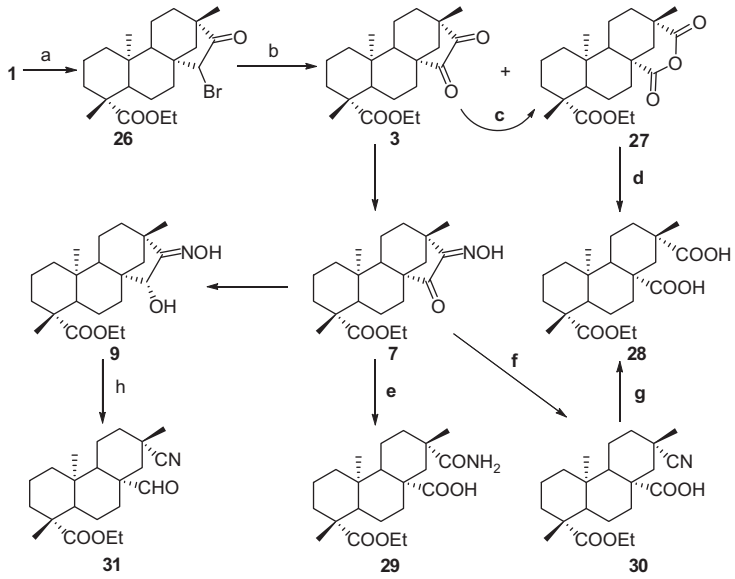
Figure 6. X-ray structure of compound **22**. Some hydrogen atoms were omitted for clarity.

After the 1,2-amino alcohol derivatives had been successfully achieved, the following efforts were focused on 1,3-amino alcohol derivatives. The synthetic routes were outlined in Scheme 3. Compound **21**, containing an α,β-unsaturated aldehyde fragment, was obtained by eliminating monomolecular H_2O from compound **11** in DBU/pyridine system. From the X-ray structure of **22** (Fig. 6)[33] we concluded that the nucleophile (pyrrolidine) could only attack the Michael acceptor (C-16) from the *endo* side and gives **22** stereoselectively. Noteworthily, this aza-Michael reaction needed a strong base (NaH), and the addition of tetrabutylammonium bromide (TBAB) could improve the yield up to 70% (20% without TBAB). Compound **12**, obtained by oximation of **11**, was converted to 1,3-amino alcohol **23** through hydrogenation catalyzed by Raney Ni in THF, and then its *N*-acetyl derivative and *N*-,*O*-diacetyl derivative were prepared. The diacetyl derivative **24** could be converted to **25** easily under a mild condition in good yield.

As the tricyclic compounds may have some kinds of bioactivities, we attempted to reconstruct the isosteviol skeleton to the corresponding tricyclic derivatives via D-ring opening reaction. Scheme 4 illustrated the synthesis of some tricyclic derivatives. The 15,16-diketone **3** could also be prepared through a bromination-oxidation process in order to avoid employing the toxic oxidant SeO_2 as aforementioned. Compound **26** was firstly prepared by a one-pot esterification-bromination from isosteviol. As expected, **3** was prepared by oxidation of **26** in DMSO/air system under reflux condition.[34] Moreover, white solid **27** was isolated from this reaction as a by-product in 20% yield, which could also be prepared from **3** through Baeyer–Villiger oxidation with excessive *m*-CPBA in high yield. Acid hydrolysis of **27** generated dicarboxylic acid **28**. As Beckmann fragmentation occur on ketoxime[35] and α-hydroxyl oxime,[36] the expected tricyclic compounds could be obtained from **7** and **9** through this reaction. Treatment of compound **7** in acetone with catalytic amount of concentrated sulfuric acid under reflux afforded compound **29** conveniently. Alternatively, treatment of compound 7 with TsCl/NaOH gave **30**, which was converted to **28** through hydrolysis of the cyano group. For α-hydroxyl oxime **9**, fragmentation in thionyl chloride provided **31** efficiently.

The MTT cell proliferation assay was used to evaluate cytotoxic activities of these new synthesized compounds[37] against four human cancer cell lines in vitro, including two esophageal carcinoma cell lines (Eca109 and EC9706), prostatic carcinoma (PC-3) and colotectal carcinoma (HCT-116). Cisplatin (DDP) was used as positive control. From the IC_{50} values summarized in Table 1[38], the 1,3-amino alcohol compound **23** showed significant cytotoxicity.

Table 1 revealed that esophageal carcinoma cells were more sensitive to the tested compounds with the IC_{50} low to 4.01 μM. The carbonyl compounds **1**, **2**, **3** and **26** without hydroxyl or amino group showed no inhibition to all cancer cell lines, but certain activities against Eca109 and EC9706 were observed when carbonyl groups were converted to hydroxyl group (**5, 10, 11** and **12**). 16-Oximido group did not improve inhibitory activity (**7**, **8**, and **9**). Noteworthily, introduction of hydroxymethyl, formyl or oximido to C-15 was beneficial for the cytotoxicity (**10**, **11** and **12**). Compound **14** with 1,2-amino alcohol subunit exhibited noteworthy activities to the four tested cells lines whereas 15-carbonyl-16-amino derivative **13** gave poor results. The results indicated that the conversion of carbonyl at C-15 and C-16 to hydroxyl was important to their bioactivities (**4** vs **5**, **13** vs **14**, **17** vs **18**, **19c** vs **20c**). Formation of phenyl and naphthyl thiourea and acylation of amino group by proline decreased cytotoxic activities greatly (**14** vs **18**, **19a**, **19b**, **20a**, **20b**). Nevertheless, 3,5-Bis(trifluoromethyl) phenyl thiourea (**20c**) showed certain effect to the cell lines. α,β-Unsaturated aldehyde **21** only had moderately cytotoxic against PC-3 cell while 16-pyrrolidine substituted compounds **22** inhibited Eca109 selectively with IC_{50} value 30.30 μM. Importantly, 1,3-amino alcohol **23** displayed the most potent anticancer agent with IC_{50} value 4.01 μM, 5.02 μM, 15.31 μM, and 12.25 μM against EC9706, Eca109, PC-3 and HCT-116, 'respectively'. Interestingly, the anti-tumor activities of **23** were superior to Cisplatin against EC9706, PC-3 and HCT-116. In addition, acylation of the 1,3-amino alcohol of **23** led to the lost of its activity (**23** vs **24**). Selective acylation of amino group caused weaker activities to the two esophageal carcinoma cells while it was inactive to PC-3 and HCT-116 cell lines (**23** vs **25**). No activity was observed for the



Scheme 4. Reagents and conditions: (a) EtBr, DMSO, KOH, 80 °C, 3 h (93%); (b) air, DMSO, reflux, 16 h, 70% of **3** and 20% of **27**; (c) *m*-CPBA, DCM, rt, 48 h (99%); (d) 10% NaOH, then HCl, (90%); (e) H_2SO_4, acetone, reflux (98%); (f) TsCl, NaOH, H_2O, 90 °C (90%); (g) H_2SO_4, H_2O, 2 h, reflux (98%); (h) $SOCl_2$, rt, 2 h (84%).

Table 1
In vitro anti-tumor activities of isosteviol derivatives against three human cancer cell lines

Compd	IC_{50}[a] (μM)				Compd	IC_{50}[a] (μM)			
	EC9706	Eca109	PC-3	HCT-116		EC9706	Eca109	PC-3	HCT-116
1	>100[b]	>100	>100	>100	**19b**	>100	>100	>100	>100
2	>100	>100	>100	>100	**19c**	>100	>100	>100	>100
3	>100	>100	>100	>100	**20a**	>100	72.28	>100	>100
4	>100	>100	>100	>100	**20b**	>100	90.88	>100	>100
5	52.08	90.26	87.56	>100	**20c**	24.07	45.82	20.63	14.35
6	>100	>100	>100	>100	**21**	>100	>100	40.34	>100
7	>100	>100	>100	>100	**22**	>100	30.30	>100	>100
8	>100	>100	>100	>100	**23**	4.01	5.02	15.31	12.25
9	>100	>100	>100	>100	**24**	>100	>100	>100	>100
10	91.63	43.33	>100	>100	**25**	21.23	13.28	>100	>100
11	24.42	26.52	>100	>100	**26**	>100	>100	>100	>100
12	56.64	17.87	>100	>100	**27**	>100	>100	>100	>100
13	97.23	>100	>100	>100	**28**	>100	>100	>100	>100
14	19.33	41.32	25.38	20.13	**29**	>100	>100	>100	>100
17	>100	>100	>100	>100	**30**	>100	>100	>100	>100
18	60.67	69.55	>100	>100	**31**	>100	>100	>100[b]	>100
19a	>100[b]	>100	>100	>100	Cisplatin	6.12	4.20	21.02	62.03

[a] Inhibitory activity was assayed by exposure for 72 h.
[b] IC_{50} Values greater than 100 μM were considered as inactive and omitted here.

amide, cyano, aldehyde, carboxyl compounds with tricyclic scaffold (**28**–**31**).

In summary, a series of polyhydric, amino alcohol and tricyclic derivatives of isosteviol were facilely synthesized and their anti-tumor activities were evaluated in vitro. The results revealed that introduction of hydroxyl and amino alcohol fragments to isosteviol were beneficial to anticancer activities while D-ring opened compounds were inactive. Esophageal carcinoma cells were more sensitive to these compounds. Especially, 1,3-amino alcohol **23** exhibited superior anticancer activities to Cisplatin with IC_{50} value 4.01 μM. Further study on optimizing the activity of amino alcohol derivatives and their polyamine derivatives based on isosteviol are in progress in our group and the results will be reported in due course.

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33. Crystallographic data (excluding structure factors) has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 866960 (compound **5**), 866959 (compound **9**), 866958 (compound **17**) and 885321 (compound **22**). Copies of the data can be obtained, free of charge, on applications to CCDC, 12 Union Road, Cambridge CB2 1EZ. UK (fax: +44 (0) 1223 336033 or E-mail: deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk).
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37. *Selected spectral data for compounds **14** and **23***: Compound **14**: white solid, FT-IR(KBr): ν 3406, 3342, 3224, 2937, 2850, 1718, 1462, 1232, 1179, 1151, 1096, 1034, 779, 629 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, ppm): δ 4.08–4.10 (m, 2H), 3.54–3.56 (d, 1H, J = 8 Hz), 2.96–2.98 (d, 1H, J = 8.0 Hz), 2.16–2.20 (m, 3H), 1.77–1.91 (m, 3H), 1.70–1.73 (d, 1H, J = 12.0 Hz), 1.60–1.64 (m, 1H), 1.50 (m, 2H), 1.35–1.43 (m, 2H), 1.24–1.28 (m, 6H), 1.17 (s, 3H), 1.10–1.12 (m, 1H), 0.97–1.04 (m, 3H), 0.94 (s, 3H), 0.88 (s, 3H), 0.84–0.80 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 177.9, 79.5, 59.9, 59.7, 58.4, 57.8, 53.2, 47.5, 43.6, 42.6, 41.3, 40.9, 38.2, 37.7, 34.3, 28.9, 26.3, 22.0, 20.6, 19.7, 14.3, 14.2; HRMS (ESI, m/z) Calcd for $C_{22}H_{38}NO_3$ $[M+H]^+$364.2846. Found: 364.2848. Compound **23**: white solid, FT-IR(KBr, cm^{-1}): ν 3432, 2944, 2849, 1718, 1631, 1558, 1460, 1381, 1327, 1233, 1156, 1063, 626. ^1H NMR (400 MHz, $CDCl_3$, ppm) δ 4.08–4.10(m, 2H), 3.53–3.54(d, 1H, J = 4.0 Hz), 3.17–3.20 (d, 1H, J = 9.0 Hz), 2.68–2.74 (t, 1H), 2.14–2.17 (d, 1H, J = 9.0), 2.04–2.08 (m, 1H), 1.91 (s, 1H), 1.69–1.81 (m, 4H), 1.55–1.65 (m, 4H), 1.32–1.42 (m, 2H), 1.24–1.27 (m, 5H), 1,15 (m, 4H), 1.04–1.04 (m, 2H), 0.95–0.98 (m, 3H), 0.90 (m, 4H), 0.76 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.3, 85.9, 60.1, 57.7, 57.0, 53.6, 47.0, 43.6, 42.7, 41.3, 39.6, 38.1, 37.9, 35.0, 33.1, 29.7, 29.0, 24.9, 21.9, 19.3, 18.9, 14.1, 13.3. HRMS (ESI, m/z) Calcd for $C_{23}H_{39}NO_3Na$ $[M+Na]^+$ m/z 400.2828. Found:400.2828.
38. *In vitro cytotoxicity study*: EC9706, Eca109, PC-3 and HCT-116 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in humidified air atmosphere of 5% CO_2. Cell cytotoxicity was assessed by MTT assay. Briefly, cells were plated into 96-well flat-bottomed plates (5×10^3 cells/well). After 24 h incubation at 37 °C, removed the culture medium and replaced with fresh medium containing the studied compounds in different concentrations to the wells, and the cells were incubated for another 72 h. Afterwards, 0.5 mg/mL MTT in PBS was added and cells were incubated for a further 4 h. Two hundred microliters of DMSO was added to each culture to dissolve the reduced MTT crystals. The MTT-formazan product dissolved in DMSO was estimated by measuring absorbance at 562 nm with a microplate reader. Then the inhibitory percentage of each compound at various concentrations was calculated, and the IC_{50} value was determined.